

Differential susceptibility of Type 1 and Type 4 gonococci to chlorhexidine (Hibitane)

SHEENA A. WAITKINS* AND I. GEARY

Department of Medical Microbiology, University of Sheffield Medical School

In recent years several reports have appeared in the literature advocating the use of chemical prophylactic measures to control the current epidemic of gonorrhoea (Lee, Utidjian, Singh, Carpenter, and Cutler, 1972; Cowan and Cree, 1973; Lee, 1974). As yet, no ideal agent has been found; we present results in this paper which suggest that Hibitane could be used as an effective prophylactic agent.

Hibitane (chlorhexidine) is a 1, 6-di C4-chlorophenyldiguanido hexane. Its salts are distinguished by their wide range of antimicrobial activity, being highly effective against both Gram-positive and Gram-negative organisms. As with other germicides, chlorhexidine is reduced somewhat in antimicrobial activity by certain organic substances notably milk, blood, and pus; however, this reduction is less marked than that observed with some of the commonly used germicides (Calman and Murray, 1956).

Chlorhexidine, when used in effective germicidal concentrations, has been reported to have very little, if any, toxic effect on animal tissues (I.C.I. Pharmaceuticals Division, 1973). Various forms of Hibitane have been used as skin disinfectant (Murray and Calman, 1955; Lowbury, Lilly, and Bull, 1960). Studies of Hibitane as a cleansing agent in wounds, burns, and skin infections have also been reported (Montgomery, 1964; Fowler, 1963; Grant, 1968), and its usefulness has been established in obstetrics and gynaecology. Calman and Murray (1956) compared Hibitane with a number of other antiseptics commonly used in midwifery and found it to have a very much safer margin. Barr (1964) stated that in general practice obstetrics swabbing the genito-urinary area with Hibitane was non-irritant and effective. Morris and McLaren (1969), in a comparative trial against commonly used deodorants, found that chlorhexidine vaginal sprays were superior

because they eliminated Gram-negative organisms which produced the odours. No spermicidal activity seems to have been reported with Hibitane used as a genital spray.

This paper presents a study of Hibitane, in the form of chlorhexidine gluconate solution, as a potential prophylactic chemical in the control of gonorrhoea. However, it soon became apparent to us that Hibitane exerted a differential bactericidal action on virulent and non-virulent gonococci; virulent strains of Type 1 were more susceptible to the killing action of Hibitane than strains of Type 4. This could prove of value as a differential marker between the gonococcal types.

Material and methods

MEDIA USED

5 per cent. lysed horse blood agar

Columbia agar base (Oxoid) 39 g., distilled water 1,000 ml. Soak for 15 min. Mix and autoclave at 121°C. for 15 min. Allow to cool to 55°C. and then add 50 ml. sterile horse blood (Oxoid) lysed by repeated freezing and thawing.

Difco G.C. medium base plus defined supplement (GCMB medium)

Difco G.C. medium base 36 g., distilled water 1,000 ml. Heat to 70°C. for 10 min. Autoclave at 121°C. for 15 min. Allow to cool to 60°C. and add 20 ml. supplement (White and Kellogg, 1965).

'Lab Lemco' broth

Lab Lemco broth granules (Oxoid) 8 g., distilled water 1,000 ml. Mix well and distribute in 5 ml. amounts. Autoclave at 121°C. for 15 min.

Serum broth

Lab Lemco broth prepared as above plus 10 per cent. inactivated horse serum adjusted to pH 4.5.

Phosphate buffered saline (PBS) pH 7.3

Dulbecco saline was prepared (Dulbecco and Vogt, 1954). Dulbecco tablets A and B Oxoid were made up as described by the manufacturers and sterilized by autoclaving.

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*Present address: Neisseria Department, Statens Serum Institute, Copenhagen

Address for reprints: Mr. I. Geary, Department of Medical Microbiology, University of Sheffield Medical School, Beech Hill Road, Sheffield, S10 2RX

Hibitane solution

Hibitane gluconate 20 per cent. solution (chlorhexidine gluconate solution B.P.) obtained from I.C.I. This was sterilized by autoclaving at 115°C. for 30 min. Final concentrations were made up in Dulbecco solution as shown in Table I.

NEUTRALIZING AGENT

Lecithin 3 g., alcohol 3 ml. (methanol), Tween-80 1 ml., Lab Lemco (prepared as above) 96 ml. The lecithin was dissolved in alcohol by heating in a water bath and the broth then added. Heating was continued until mixing was complete. Tween-80 was then added and the solution was sterilized by autoclaving at 115°C. for 15 min. Just before use the neutralizing agent was diluted 1:2 with sterile distilled water.

TEST ORGANISMS

All strains of *Neisseria gonorrhoeae* were isolated from patients attending the Sheffield V.D. Clinic and cultured initially on 5 per cent. lysed horse blood agar with vancomycin (3 µg./ml.). They were identified by Gram's stain, a positive oxidase reaction, and the ability to ferment glucose but not maltose or sucrose. Fermentation tests were performed using a modified carbohydrate medium containing Difco GC medium base with 1 per cent. sugar concentrations (Flynn and Waitkins, 1972). After one subculture had shown the strains to be pure they

were then selected for colonial Types 1 and 4 (Kellogg, Peacock, Deacon, Brown, and Pirkle, 1963).

PREPARATION OF ORGANISMS FOR EXPERIMENTS

N. gonorrhoeae were grown in Difco G.C. medium base for 18 hrs at 37°C. in a 5 per cent. CO₂ atmosphere. Colony types were checked using a Zeiss-stereoscan IV and the organisms were then scraped off and washed three times in warmed Dulbecco phosphate saline pH 7.3. The deposit was re-suspended in 10 ml. Dulbecco saline and the turbidity of suspensions measured on an E.E.L. nephelometer. The bacterial counts were estimated by reference to previously prepared graphs, to give a standard curve of approximately 1×10^4 organisms/ml. These counts were confirmed by the Miles and Misra method on 5 per cent. lysed horse blood agar (Miles, Misra, and Irwin, 1938).

EXPERIMENTAL PROCEDURE

Hibitane solution

Dilutions of Hibitane gluconate in PBS were made from 1/1,000 to 1/10,000.

Experiment

The experimental procedure was that shown diagrammatically in the Figure. To each dilution of Hibitane solution was added 0.5 ml. bacterial suspension. The mixture was allowed to react for 2½ min. before being stopped by the

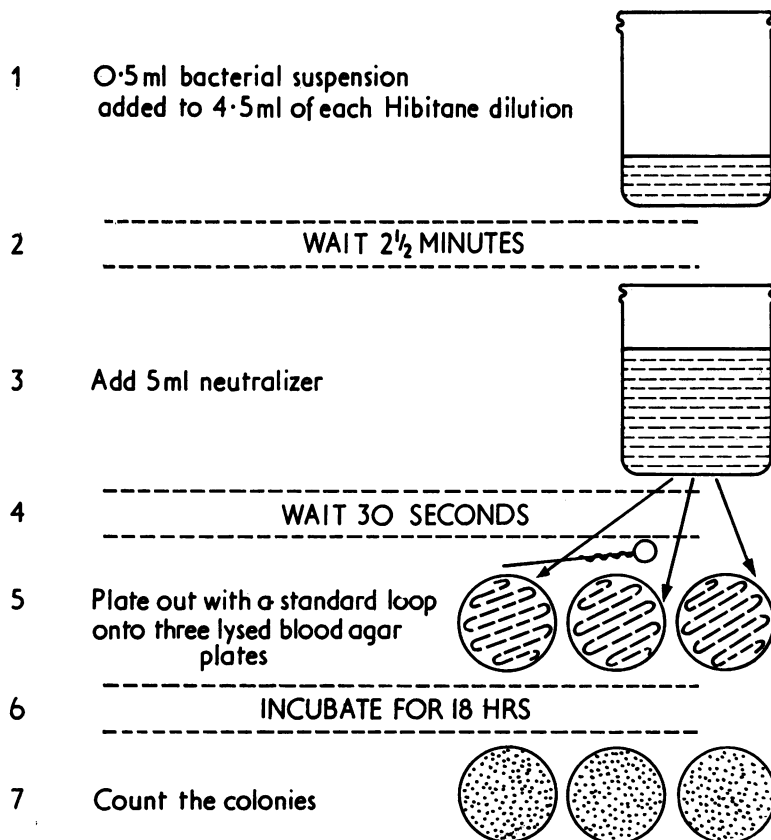


FIGURE
Experimental procedure

addition of 5 ml. of neutralizer. After 30 sec. the final mixture was plated out in triplicate onto lysed horse blood agar using a standard platinum loop. To ensure that the neutralizer did not itself inhibit the organisms, a control of gonococci plus neutralizer was included and further Miles and Misra counts were carried out as before for comparison with those of the initial inoculum (Miles and others, 1938).

Results

Table I illustrates the percentage kill of *N. gonorrhoeae* by various dilutions of Hibitane. In this first experiment the strains used were unselected and both Types 1 and 4 were randomly distributed among the numbers sampled. As can be seen from the results, there was a reasonably high percentage kill in the cases of all strains; however, strains T5, 24, and 26 were exceptionally sensitive to the action of Hibitane giving 100 per cent. kill dilutions as low as 1/5,000, an extremely low concentration for this antiseptic. On further investigation all of these strains were found to be Type 1 and consequently a comparison of the action of Hibitane on Type 1 and 4 variants of seven strains was undertaken. The findings are shown in Table II. The dilutions of Hibitane were varied from 1/1,000 to 1/10,000, and the sensitivity of Types 1 and 4 compared. It is interesting to note that in almost all instances Type 1 variants were much more sensitive to the killing action of Hibitane than were Type 4 variants.

The foregoing results were obtained using suspending medium at pH 7.3. For Hibitane to be an effective bactericidal agent for prophylaxis, it must

be equally effective at the normal lower vaginal pH of 4.0 to 5.0. Table III gives the results of a further comparison of Types 1 and 4 using as diluent serum broth at pH 4.5. Very little impairment of the action of Hibitane was observed at the lower pH and Type 1 again appeared to be more sensitive than Type 4.

Discussion

Gonococci are extremely sensitive to the bactericidal action of Hibitane gluconate solution, even at low concentrations. Furthermore, virulent colonies of Type 1 appear to be much more susceptible to this action than those of the avirulent Type 4.

These morphological differences were shown to be associated with variations in virulence of the gonococcal colonies by Kellogg and his colleagues who demonstrated that colonies of Types 1 and 2 would cause a urethral discharge in male volunteers, while those of Types 3 and 4 would not (Kellogg, Peacock, Deacon, Brown, and Pirkle, 1963). Until recently, there was no known biochemical or cytological explanation for these morphological dissimilarities. However, in 1971, Swanson showed that pili could be found in greater numbers on Types 1 and 2 gonococci, whereas Types 3 and 4 possessed very few of these structures (Swanson, Kraus, and Gotschlich, 1971).

More recently, Cheng, Kwapinski, and Ronald (1974) demonstrated that Type 1 colonies contained a unique protein which was extremely toxic to chick embryos. This protein could not be found in colonies

TABLE I Percentage kill of *N. gonorrhoeae* at various concentrations of 20 per cent. Hibitane gluconate solution

| Strain no. | Initial inoculum | Percentage kill by Hibitane (final concentrations) | | | | | Control (orgs./ml. recovered) |
|------------|-----------------------|--|---------|---------|---------|---------|-------------------------------|
| | | 1/1,000 | 1/2,000 | 1/3,000 | 1/4,000 | 1/5,000 | |
| 42 | 1 × 10 ⁴ | 100 | 99.7 | 99.5 | 98.2 | 96.0 | 1 × 10 ⁴ |
| T6 | 1 × 10 ⁵ | 100 | 99.41 | 93.6 | 87.2 | 86.1 | 1 × 10 ⁵ |
| 1 | 15 × 10 ³ | 100 | 99.89 | 99.73 | 96.4 | 94.0 | 13 × 10 ³ |
| 3 | 54 × 10 ⁴ | 99.1 | 96.2 | 95.88 | 93.2 | 89.9 | 62 × 10 ⁴ |
| 55 | 13 × 10 ⁴ | 99.8 | 99.6 | 99.3 | 98.9 | 92.0 | 11 × 10 ⁴ |
| 41 | 36 × 10 ⁴ | 98.7 | 97.6 | 95.1 | 94.2 | 93.1 | 14 × 10 ⁴ |
| 62 | 9 × 10 ³ | 100 | 100 | 99.1 | 98.0 | 94.9 | 1 × 10 ³ |
| 33 | 1 × 10 ⁴ | 100 | 99.8 | 99.7 | 99.6 | 99.7 | 95 × 10 ⁴ |
| T5 | 39 × 10 ⁵ | 100 | 100 | 100 | 100 | 100 | 41 × 10 ⁵ |
| T4 | 101 × 10 ⁴ | 100 | 99.4 | 97.1 | 96.0 | 95.3 | 98 × 10 ⁴ |
| T3 | 54 × 10 ⁴ | 100 | 99.8 | 99.6 | 98.7 | 96.9 | 57 × 10 ⁴ |
| 21 | 14 × 10 ⁴ | 99.8 | 99.5 | 98.9 | 98.1 | 97.4 | 13 × 10 ⁴ |
| 22 | 44 × 10 ³ | 99.9 | 99.6 | 99.3 | 99.2 | 99.8 | 56 × 10 ³ |
| 23 | 42 × 10 ³ | 100 | 98.5 | 84.0 | 72.0 | 50.0 | 42 × 10 ³ |
| 24 | 36 × 10 ⁵ | 100 | 99.8 | 87.5 | 96.8 | 95.4 | 15 × 10 ⁵ |
| 25 | 4 × 10 ⁴ | 100 | 100 | 100 | 100 | 100 | 9 × 10 ⁴ |
| 26 | 6 × 10 ⁴ | 100 | 100 | 100 | 100 | 100 | 13 × 10 ⁴ |
| 27 | 54 × 10 ⁴ | 100 | 100 | 99.8 | 99.2 | 97.9 | 60 × 10 ⁴ |
| 28 | 5 × 10 ³ | 100 | 99.81 | 96.2 | 98.7 | 95.1 | 1 × 10 ³ |
| 29 | 9 × 10 ⁵ | 100 | 100 | 100 | 98.1 | 96.5 | 18 × 10 ⁵ |
| 30 | 89 × 10 ⁴ | 99.9 | 98.3 | 96.1 | 97.3 | 94.1 | 1 × 10 ⁴ |
| 31 | 4 × 10 ⁴ | 100 | 100 | 99.3 | 99.1 | 97.9 | 3 × 10 ⁴ |
| 32 | 3 × 10 ³ | 100 | 99.9 | 100 | 99.6 | 99.0 | 4 × 10 ³ |
| 34 | 21 × 10 ⁵ | 100 | 98.3 | 89.4 | 73.0 | 51.9 | 25 × 10 ⁵ |
| 35 | 9 × 10 ³ | 100 | 99.1 | 99.1 | 96.6 | 95.3 | 3 × 10 ³ |
| 36 | 21 × 10 ⁴ | 100 | 98.2 | 96.5 | 95.4 | 93.2 | 28 × 10 ⁴ |

TABLE II *Effect of various dilutions of 20 per cent. Hibitane gluconate solution on gonococcal Types 1 and 4*

| Strain no. | Type | Initial inoculum | Percentage kill by Hibitane (concentrations) | | | | | | Control (Orgs/ml. recovered) |
|------------|------|------------------|--|---------|---------|---------|---------|----------|------------------------------|
| | | | 1/1,000 | 1/3,000 | 1/5,000 | 1/7,000 | 1/9,000 | 1/10,000 | |
| 9 | 1 | 7×10^4 | 100 | 100 | 100 | 100 | 99.5 | 97.3 | 7×10^4 |
| | 4 | 4×10^4 | 100 | 99.1 | 98.7 | 96.8 | 95.4 | 89.1 | 4×10^4 |
| 10 | 1 | 10×10^4 | 100 | 100 | 100 | 100 | 100 | 99.8 | 11×10^4 |
| | 4 | 14×10^4 | 100 | 99.8 | 97.4 | 95.8 | 93.2 | 90.1 | 16×10^4 |
| 12 | 1 | 10×10^4 | 100 | 100 | 100 | 100 | 100 | 100 | 16×10^4 |
| | 4 | 14×10^4 | 99.1 | 96.4 | 98.1 | 95.1 | 91.8 | 86.1 | 1×10^4 |
| 25 | 1 | 6×10^4 | 100 | 100 | 100 | 100 | 99.1 | 98.1 | 13×10^4 |
| | 4 | 9×10^4 | 100 | 99.8 | 97.0 | 89.6 | 84.2 | 81.9 | 1×10^4 |
| 26 | 1 | 13×10^5 | 100 | 100 | 100 | 100 | 99.8 | 99.8 | 12×10^5 |
| | 4 | 13×10^5 | 100 | 99.8 | 84.2 | 46.0 | 40.1 | 36.9 | 16×10^5 |
| 11 | 1 | 14×10^4 | 100 | 100 | 99.1 | 96.1 | 93.4 | 90.2 | 16×10^4 |
| | 4 | 6×10^5 | 100 | 100 | 100 | 99.8 | 96.7 | 96.0 | 5×10^5 |
| 16 | 1 | 13×10^5 | 100 | 100 | 100 | 100 | 100 | 100 | 14×10^5 |
| | 4 | 15×10^4 | 99.9 | 98.3 | 96.5 | 95.9 | 94.3 | 92.1 | 36×10^4 |

TABLE III *Effect of various dilutions of 20 per cent. Hibitane gluconate solution on gonococcal Types 1 and 4 using Lab Lemco and horse serum at pH 4.5*

| Strain no. | Type | Initial inoculum | Percentage kill by Hibitane (concentrations) | | | | | | Control (Orgs/ml. recovered) |
|------------|------|------------------|--|---------|---------|---------|---------|----------|------------------------------|
| | | | 1/1,000 | 1/3,000 | 1/5,000 | 1/7,000 | 1/9,000 | 1/10,000 | |
| 9 | 1 | 15×10^4 | 100 | 100 | 100 | 100 | 97.8 | 95.2 | 12×10^4 |
| | 4 | 16×10^4 | 100 | 99.8 | 97.5 | 95.4 | 95.4 | 88.9 | 19×10^4 |
| 10 | 1 | 10×10^3 | 100 | 100 | 100 | 99.5 | 95.9 | 95.1 | 13×10^3 |
| | 4 | 15×10^3 | 100 | 100 | 99.2 | 96.3 | 94.8 | 90.1 | 15×10^3 |
| 12 | 1 | 1×10^3 | 100 | 100 | 100 | 100 | 100 | 100 | 4×10^3 |
| | 4 | 3×10^3 | 100 | 99.8 | 97.0 | 89.5 | 84.8 | 83.0 | 1×10^3 |
| 25 | 1 | 6×10^5 | 100 | 100 | 100 | 99.4 | 98.9 | 97.0 | 1×10^5 |
| | 4 | 9×10^4 | 99.8 | 96.2 | 80.2 | 53.0 | 45.1 | 36.8 | 1×10^4 |
| 26 | 1 | 4×10^4 | 100 | 100 | 100 | 99.4 | 98.6 | 98.2 | 8×10^4 |
| | 4 | 5×10^4 | 100 | 99.8 | 90.0 | 89.3 | 86.1 | 85.0 | 1×10^4 |
| 11 | 1 | 11×10^4 | 100 | 100 | 100 | 100 | 96.4 | 90.9 | 9×10^4 |
| | 4 | 12×10^4 | 100 | 99.9 | 99.4 | 98.6 | 97.1 | 95.0 | 9×10^4 |
| 16 | 1 | 13×10^3 | 100 | 100 | 100 | 100 | 100 | 98.1 | 14×10^3 |
| | 4 | 15×10^3 | 99.9 | 98.4 | 96.3 | 96.0 | 94.3 | 90.0 | 36×10^3 |

of Type 4 organisms. Waitkins (1974) showed that Types 1 and 2 would haemagglutinate human red blood cells, while Type 4 would not. The author concluded that the variation in haemagglutination power was due to the absence of any pili on the avirulent Type 4. To date these are the only distinguishing factors, other than virulence, between Kellogg's different morphological types.

Our findings indicate that Hibitane gluconate solution has a differential action on Type 1 and Type 4 gonococci. Table II illustrates the varying bactericidal effect that Hibitane gluconate has on both Types 1 and 4 of the same strains of gonococci. Although results from testing only seven strains are reported, we found the same differential effect between Types 1 and 4 in over fifty other strains tested. Furthermore,

the killing effect of Hibitane was not greatly altered when Lab Lemco plus horse serum was used as diluent. These results would suggest that, at least quantitatively, there is a detectable difference in susceptibility between Kellogg's Types 1 and 4 using suitable test dilutions of Hibitane gluconate solution.

These findings suggest that Hibitane might be used as a chemical prophylactic agent in the control of gonorrhoea; however, *in vivo* verification of its effectiveness would be required and like all topical agents there remains the problem of ensuring that it reaches all organisms in adequate concentration.

Summary

Virulent Type 1 gonococci have been shown to be much more susceptible to the killing action of

Hibitane gluconate than the avirulent Type 4. Those exploring the possibility of genital prophylaxis as a means of controlling gonorrhoea might find Hibitane worthy of consideration.

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